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Acute effects of TCDD administration: special emphasis on testicular and sperm mitochondrial function

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ABSTRACT

Objective: The goal of this study was to verify if 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) could have any effect on male germ cells mitochondria and in this way add new insights in how male reproductive alterations observed in other studies occur. **Methods:** *In vivo* and *in vitro* approaches using rat testis and human sperm as models were employed to evaluate TCDD effects on testicular and sperm mitochondria after 24 h of exposure. **Results:** Testicular mitochondria from TCDD-treated rats presented no differences in the bioenergetic parameters monitored except for a significantly higher electric membrane potential in the presence of ADP, corroborated when TCDD was directly added to testicular mitochondria from untreated rats. Nevertheless, sperm mitochondrial membrane potential, motility, viability, capacitation and acrosomal integrity did not change after TCDD treatment. Moreover, only few sperm cells exposed to TCDD increased their intracellular Ca^{2+} concentration. **Conlusions:** TCDD can interact directly with rat testicular mitochondria inducing small changes. This effect, however, does not seem to occur in human sperm or it may be insufficient to induce significant alterations as observed by the maintenance of sperm function.

1. Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs), commonly identified as dioxins, are a well-known family of persistent organochlorine compounds formed as products and/or by-products derived from industrial or combustion systems[1,2] that bio-accumulate in the food chain and exert their toxicity by binding to the aryl hydrocarbon receptor (AhR). As other so-called endocrine disruptors, PCDDs interference with hormonal action - mimicking or antagonizing the action of endogenous hormones - may be related with the decline in human and wildlife semen quality reported worldwide[3-6]. However, the existing data in humans are inconsistent: while some failed to observe different levels of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, the prototypical dioxin) in adipose tissue of both fertile and infertile men[7] or any association between exposure and pregnancy outcome[8,9], others found altered levels of sexual hormones in men exposed to TCDD either by food poisoning[10] or by occupational exposure[11]. However, most of the information on the toxic effects of dioxins originates from studies performed with the Seveso population, exposed to high amounts of TCDD after an explosion in a trichlorophenol manufacturing plant in 1976. In this population, TCDD was associated with a lower male/female sex ratio[12] and the likelihood of fathering a female child was augmented with increasing serum TCDD concentrations from the fathers, particularly when they were, at the time of the accident, younger than 19 years old[1]. This sex ratio reduction was further observed in Russian pesticide workers exposed to TCDD[13], but this finding has not been reported by others[9]. More recently, Mocarelli and co-workers found that men exposed to TCDD during childhood presented abnormal sperm concentration, motility and hormone levels even 22 years after exposure, whereas the ones exposed during puberty or adulthood showed an increase or no differences in relation to controls, respectively[2]. The multitude and age variation of the effects observed after TCDD exposure make it difficult to determine if all these alterations are due to the endocrine

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deregulation effect or if dioxins, particularly TCDD, may act on the reproductive system through alterations in some other pathways. In fact, Simanainen and colleagues suggested that TCDD may act through other pathways, after describing that three different strains of rats with different susceptibilities to TCDD-induced toxicity presented the same testosterone levels but variable levels of spermatogenesis derangement after exposure[14]. The same was observed in the Mocarelli study where, although the same abnormalities in hormone levels were detected in pre-pubertal and pubertal groups 22 years after TCDD exposure, the semen parameters were divergent[2].

Mitochondria are the main cellular energy producers but are also involved in cell Ca^{2+} homeostasis, intermediate metabolism free radical generation and cell death. Such huge range of functions also makes these organelles potential targets of drugs and, in particular, environmental contaminants[15,16].

Some of the studies performed to evaluate the toxicity and carcinogenic potential of TCDD have focused on mitochondrial dysfunction and revealed, among other things, decreased mitochondrial membrane potential (MMP)[17,18], increased reactive oxygen species (ROS) production[19-21], altered mitochondrial DNA copy number[22] and decreased expression of nuclear-encoded mitochondrial respiratory chain subunits[23]. Some of these effects arose without any alterations on gene expression, as observed in *in vitro* studies of mitochondrial fractions[24,25], indicating that mitochondria may be one of the first targets as well as effectors of TCDD toxicity. Several alterations found in the male reproductive system following TCDD exposure also point towards mitochondrial deregulation. Indeed, TCDD caused oxidative stress in rat epididymis[26], testis[27-33], Sertoli cells[34] and epididymal sperm[26,35]. Moreover, an acute exposure to TCDD caused MMP loss due to high ROS levels in mouse sperm[36]. Also, in accordance with a role of altered mitochondria physiology and signaling on the development of TCDD toxicity is the up-regulation of BAX and p53 expression in rat testis after TCDD treatment[37].

The present work aims at testing the relevant hypothesis that mitochondria are effectors of TCDD toxicity in the male reproductive system. For that, we used *in vivo* and *in vitro* approaches to test mitochondrial functionality in both testis and sperm in the presence of TCDD. Since we cannot perform *in vivo* experiments in men, we chose an experimental rat model (Wistar Han rats) that is thought to have a similar sensitivity to TCDD, with a binding activity of 1.07-1.34 nmol/L[38], similar to the one observed with human AhR (1.58 nmol/L[39]). Furthermore, we have used testis, and not liver, to investigate mitochondrial alterations, given the functional bioenergetic differences in those organelles we previously reported when comparing those organs[40]. Moreover, as human sperm expresses the AhR receptor[41] and several reports suggests that environmental toxicants may induce non-genomic actions and thus contact directly with pre-existing pathways[42-44], we went on to evaluate the effect of an acute exposure to TCDD in human sperm mitochondria and further analyze other functional parameters such as motility, capacitation and acrosomal integrity.

2. Material and methods

2.1. Reagents

All reagents were supplied by Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. A 99% chemical pure TCDD (LGC Standards, Barcelona, Spain) was dissolved in dimethyl sulfoxide (DMSO) to a final stock concentration of 66.7 μg/mL.

2.2. Experiments using isolated testicular mitochondria

2.2.1. Animal care

All experiments involving animals were conducted in accordance with the European convention for the protection of vertebrate animal used for experimental and other scientific purposes (CETS no. 123 of 18 March 1986 and 2005 revision, and the Commission Recommendation of 2007 C (2007) 2525). Rats were maintained in proper environmental requirements including room temperature set at 22 °C, relative humidity at 45%-65%, ventilation with 15-20 changes/h, 12 h light/dark cycle, noise level < 55 dB, and *ad libitum* access to standard rodent food (4RF21 GLP certificate, Mucedola, Italy) and acidified water (at pH 2.6 with HCl, in order to prevent bacterial growth). All animals were acclimated 10-14 d prior to experiment initiation.

Ten-week-old male Wistar rats [*Rattus norvegicus albinus*, Wistar Han IGS; outbred strain from Charles River (France)] were injected intraperitoneally with TCDD (50 μg/kg of body weight) or with an equivalent volume of DMSO alone (controls), 24 h before sacrifice. Although a seemingly high dose, it is still very far from the LD_{50} for Wistar Han rats (>10 000 μg/kg of body weight - homozygous) in acute assays[45] and was used in studies that evaluated the spermatogenic alterations after TCDD exposure in mice[46]. Untreated animals were used for the *in vitro* studies. The rats were euthanized by cervical dislocation and decapitation and testes were retrieved and placed in chilled isolation medium containing 250 mmol/L sucrose, 0.2 mmol/L EGTA, 0.1 mmol/L EDTA, 5 mmol/L HEPES (pH 7.4 with KOH) and 1 g/L fatty acid free BSA.

2.2.2. Testicular mitochondria isolation

Isolation of testicular mitochondria was performed according to Mota *et al*[40]. Briefly, finely minced testes tissue was suspended in isolation medium and homogenized with a tightly fitted Potter-Elvjjem homogenizer (Teflon:glass pestle) and then centrifuged at 2 500 r/min for 10 min (Sorvall RC-5C, Plus, SS 34 rotor, 4-8 °C). The resulting supernatant was centrifuged at 10 000 r/min for 10 min. The pellet (mitochondrial fraction) was resuspended twice in washing medium [250 mmol/L sucrose, 5 mmol/L HEPES-KOH (pH 7.4)] and repelleted at 10 000 r/min for 10 min. Mitochondrial protein content was determined by the biuret method, calibrated with BSA.

2.2.3. Mitochondrial transmembrane potential and ATP synthase activity

Mitochondrial transmembrane potential ($\Delta\psi$) and ATP synthase activity were estimated as previously described[40] with some adaptations. Tetraphenylphosphonium (3 μmol/L TPP^+) was added to the reaction medium [65 mmol/L KCl, 125 mmol/L sucrose, 10 mmol/L Tris, 20 mol/L EGTA, 2.5 mmol/L KH_2PO_4 (pH 7.4), 1 g/L free fatty acid BSA at 30 °C] supplemented with 3 μmol/L rotenone (Complex I inhibitor) and 5 mmol/L succinate (substrate for complex II). The reaction was started with addition of isolated mitochondria (0.8 mg of protein content per milliliter of medium). ADP-induced depolarization was elicited by adding ADP (25-100 μmol/L).

In the *in vitro* studies, $\Delta\psi$ and ATP synthase activity were determined simultaneously using a pH electrode, a reaction medium with low buffering capacity (0.5 mmol/L Tris) and by calibrating the system with a known amount of 10 mmol/L HCl at the end of each experiment. TCDD (1 μmol/mg of mitochondrial protein) was added to the energized mitochondria and left to incubate for 3 min in order to visualize effects on maximum $\Delta\psi$. Control assays were performed by adding the same amount of vehicle – DMSO – to the energized mitochondria.

2.2.4. Measurement of mitochondrial oxygen consumption

Oxygen consumption of isolated testis mitochondria was monitored polarographically with a Clark-type oxygen electrode as described[40]. In the *in vitro* studies, TCDD (1 μmol/mg of mitochondrial protein) was added 2 min after the mitochondrial fraction and left to incubated for 3 min. ADP (25-100 μmol/L) was added to induce state 3 respiration. After reaching a stable state (State 4 respiration), oligomycin (2 μg/mL) was added followed by *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP, 1 μmol/L), an uncoupler, to determine the maximum oxygen consumption.

2.3. Experiments using human sperm

2.3.1. Human normozoospermic samples

Fresh sperm samples were obtained from patients undergoing routine semen analysis or fertility treatments in the Human Reproduction Service at University Hospitals of Coimbra, Portugal. Patients signed informed consent forms and samples were used in accordance with the appropriate Ethical and Internal Review Board of the participating institution. Sperm samples were obtained by masturbation after 3-5 d of sexual abstinence and routine seminal analysis was performed according to the World Health Organization guidelines (WHO)[47]. Spermatozoa were prepared by density gradient centrifugation (Isolate® Sperm Separation Medium, Irvine Scientific, CA, USA) and subsequently washed and incubated for at least 3 h at 37 °C and 5% (v/v) CO_2 in a capacitating medium (Origio Sperm Preparation Medium, Medicult, Jyllinge, Denmark). For this purpose, only samples with no leucocytes (or any other round cells) and standard sperm concentration, motility and morphology values above WHO reference lower limits were used[47]. Sperm cells (10^6/mL) were then exposed to 1 nmol/L[36] or 1 μmol/L TCDD for 24 h at 37 °C and 5% (v/v) CO_2 in a saline phosphate buffer (PBS; Invitrogen, Paisley, UK) containing 1% (v/v) penicillin/streptomycin, 0.9 mmol/L $CaCl_2$, 0.5 mmol/L $MgCl_2$, 5 mmol/L D-glucose, 10 mmol/L Na-lactate, 1 mmol/L Na-pyruvate and 3 g/L BSA (pH 7.2-7.4) according to our previously described long-term culture system[48]. Controls were performed by adding 0.5% (v/v) DMSO to the medium.

2.3.2. Motility, viability and mitochondrial function

Motility was assessed by phase contrast microscopy. Two hundred spermatozoa were scored in four different fields and results were expressed as total motility (progressive and *in situ* motility).

To assess both viability and MMP, spermatozoa were incubated with 100 nmol/L SYBR14 and 240 nmol/L propidium iodide (kit LIVE/DEAD; Molecular Probes, Oregon, USA) coupled with 2 μmol/L JC-1 (5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide; Molecular Probes), respectively, for 20 min at 37 °C, in the dark[49]. Slides were observed using a Zeiss Axioplan 2 Imaging fluorescence microscope (Carl Zeiss, Göttingen, Germany) equipped with a triple band-pass filter and two hundred spermatozoa were observed. Results were expressed as percentage of viable sperm (head with green fluorescence) and with high functional mitochondria (High MMP; orange midpiece).

2.3.3. Capacitation status and acrosomal integrity

After density gradient centrifugation, spermatozoa were allowed to capacitate for 24 h under TCDD exposure in the above PBS-based medium supplemented with 25 mmol/L bicarbonate. Capacitation was then evaluated by the detection of phosphorylated tyrosines using a rabbit anti-human phosphotyrosine polyclonal antibody (Zymed, South San Francisco, CA, USA) as described elsewhere[50]. Two hundred spermatozoa were evaluated through fluorescence microscopy, with those labeled in the entire tail considered positive.

Acrosomal integrity was assessed using Pisum Sativum Agglutinin – an acrosomal content marker – coupled with fluorescein isothiocyanate (PSA-FITC). Acrosome reaction (AR) was induced by incubation with 3.2 μmol/L progesterone for 1 h at 37 °C. Spermatozoa were fixed with 2% (v/v) formaldehyde in PBS for 40 min, permeabilized (1% (v/v) Triton X-100 in PBS; 20 min) and blocked with 1 g/L BSA and 100 mmol/L glycine in PBS for 30 min at room temperature. Cells were then incubated with PSA-FITC (1:200) for 1 h at 37 °C and washed (0.1% Triton X-100 in PBS) for 15 min. A final labeling with DAPI (4,6-diamino-2-phenyl-indole; Molecular Probes) was used to counterstain spermatozoa nuclei. The proportion of spermatozoa with intact acrosome was scored through fluorescence microscopy and two hundred spermatozoa were counted in different fields.

2.3.4. Intracellular calcium concentration ($[Ca^{2+}]_i$) measurements by single-cell imaging

Sperm samples from human healthy donors recruited at Biosciences School, University of Birmingham, United Kingdom, were obtained accordingly with the Human and Embryology Authority Code of Practice and all donors gave informed consent to the work. Samples were obtained by masturbation after 3-5 d of sexual abstinence and were analyzed in accordance with WHO criteria[47]. Motile cells were carefully removed after 1 h of swim-up and were allowed to capacitate in supplemented Earle's balanced salt solution medium (sEBSS) with 3 g/L BSA at 37 °C and 5% (v/v) CO_2. Samples were then used to perform single-cell imaging. Intracellular Ca^{2+} measurements were performed as previously described[51]. Briefly, 4×10^6/mL capacitated sperm were loaded with 10 μmol/L Oregon Green BAPTA-1AM, a Ca^{2+} marker, for 1 h at 37 °C and 5% (v/v) CO_2 in a purpose-built, perfusable, imaging chamber, where the lower surface was a coverslip previously coated with 10 g/L air-dried poly-D-lysine solution. Cells adhered to the poly-D-lysine-coated area and were then observed in a Nikon TE200 inverted microscope. The chamber was connected to the perfusion apparatus, and any loose cells and extracellular dye were removed by perfusion of the chamber with sEBSS before starting recording. The experimental protocol took approximately 15 min and consisted of 3-minute perfusion with sEBSS, followed by perfusion with DMSO (3.5 min), TCDD (3.5 min), sEBSS (3.0 min), and 3.2 μmol/L progesterone

to determine if sperm were responding properly to physiological stimuli (1–2 min), all diluted in sEBSS medium, in a dark room at 25 °C with a perfusion rate of 0.4 mL/min. Real time images of all experiments were recorded by an acquisition software platform, IQ (Andor Technology, Belfast, UK), at intervals of 2.5 s. At each TCDD concentration, sperm cells were analyzed individually towards the vehicle in a total of five independent experiments from five different donors (n=100 sperm cells/experiment). Using the same settings, experiments with DMSO were also performed for 7 min (total amount of time in which spermatozoa were exposed to both DMSO and TCDD) to rule out the hypothesis that DMSO may be the one altering $[Ca^{2+}]_i$ throughout time.

2.4. Statistical analysis

Statistical analysis was carried out using the SPSS version 13.0 software for Windows (SPSS Inc., Chicago, IL, USA). All values are expressed as mean±SEM. All variables were checked for normal distribution and paired t-test or the related samples Wilcoxon signed rank test (for non-normal variables) were performed to compare control and TCDD-treated animals/mitochondria/sperm. P<0.05 was considered significant.

3. Results

3.1. Bioenergetic parameters of isolated testicular mitochondria from control and TCDD-treated animals

3.1.1. Electrochemical gradient measurements

There were no differences in the main bioenergetics parameters measured with the TPP^+ electrode indicating a clear capacity of testicular mitochondria from TCDD-treated animals to continue to regulate the electrochemical gradient essential for ATP production and ROS generation (Table 1). An exception, however, was uncovered when mitochondria were incubated with ADP. In the *in vivo* experiments, the depolarization potential elicited by adding ADP to testicular mitochondria from TCCD-treated animals was significantly higher (P=0.042) than that in the control animals, and there was a tendency for increased ADP-induced depolarization (Maximum potential-Depolarization potential, P=0.056). This was later confirmed using the *in vitro* assays, when mitochondria were exposed to a higher concentration of TCDD, albeit for a shorter period of time. ADP-induced depolarization potential was significantly reduced in the TCDD-treated mitochondria (P=0.001).

The flow of protons through the ATP synthase, monitored with a pH electrode in the presence of a reaction medium with low buffering capacity, presented no differences between control and TCDD-treated isolated mitochondria (Figure 1).

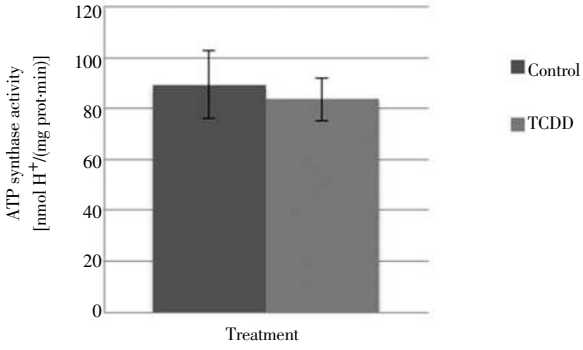

Figure 1. ATP synthase activity in isolated mitochondria treated with DMSO (control) or TCDD (1 μmol/mg of mitochondrial protein) after adding ADP (100 μmol/L) (n=5).
P>0.05 between control and treatment group.

3.1.2. Oxygen consumption by isolated testicular mitochondria

After calculation of the respiratory control ratio (RCR: St3/St4) and ADP/O (nmol ADP phosphorylated per natom oxygen consumed) ratio according to Chance and Williams[52], no significant differences in oxygen consumption, either in the *in vivo* or in the *in vitro* experiments, in all conditions evaluated, were observed (Table 2).

Table 1
Variations in mitochondrial membrane potential determined using the TPP^+ electrode.

Trial	Treatment (50 μg/kg b.w.)	Maximum potential (mV)	Depolarizing potential (mV)	Repolarizing potential (mV)	ADP-induced depolarization (mV)	Lag phase (s)
In vivo	Control	−217.5±0.8	−192.3±2.1	−212.8±0.6	−25.1±2.2	56.9±12.2
(n=8)	TCDD	−217.3±1.1	−194.3±1.6*	−213.8±1.0	−23.1±1.7	60.5±11.7
In vitro	Control	−216.7±1.4	−196.7±1.0	−210.8±1.3	−20.0±2.0	74.5±9.6
(n=11)	TCDD	−215.9±1.6	−198.1±1.2*	−211.1±1.0	−17.9±2.3**	87.0±11.1

Results represent the mean±SEM. *P<0.05 between control and respective treatment group and **P<0.01 between control and respective treatment group.

Table 2
Isolated mitochondria oxygen consumption measured using a Clark-type electrode.

Trial	Treatment	State 3 respiration	State 4 respiration	Respiration oligomycin	Uncoupled respiration	ADP/O	RCR
In vivo	Control	63.3±8.2	38.7±5.2	13.8±3.1 (n=3)	124.3±8.7 (n=3)	2.0±0.5	1.6±0.2
(n=8)	TCDD	62.1±7.4	37.4±4.1	13.7±2.5 (n=3)	124.1±13.3 (n=3)	1.9±0.4	1.6±0.1
In vitro	Control	68.5±5.0	36.3±1.9	7.5±1.4 (n=5)	127.1±10.4 (n=5)	1.4±0.1	1.9±0.1
(n=11)	TCDD	65.5±6.0	36.6±2.0	8.9±1.6 (n=5)	125.5±10.6 (n=5)	1.4±0.1	1.8±0.1

Oxygen consumption [natomO/(mg prot·min)] was measured in the presence of ADP (25–100 μmol/L), olygomycin [ATP synthase inhibitor (subunit Fo: 2 μg/mL)] or an uncoupler (1 μmol/L FCCP). Results represent the mean±SEM. P>0.05 between control and treatment group.

3.2. Effects of TCDD exposure on human sperm

3.2.1. Sperm motility, viability and MMP

After 24 h of exposure, TCDD failed to induce any adverse effect on sperm motility and survival, as shown by the inexistence of any statistical significance at both low and high concentrations (Table 3). Moreover, when assessing MMP by fluorescence microscopy, we were not able to detect any differences even at the highest dose tested (Figure 2).

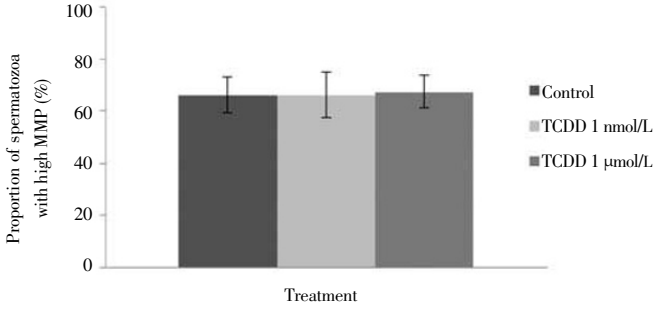

Figure 2. Proportion of human spermatozoa with high MMP after 24 h of exposure to TCDD at 37 °C and 5% (v/v) CO_2.
Two hundred sperm cells were observed in each sample (n=6) and results represent mean±SEM. P>0.05 between control and treatment groups.

3.2.2. Sperm capacitation and acrosomal integrity

Since spermatozoa need to capacitate and undergo AR in order to fertilize an oocyte, we decided to evaluate these two important parameters. However, there were no significant differences between fully capacitated sperm detected by tyrosine phosphorylation after TCDD and DMSO treatment (Table 3). Furthermore, a 24-hour exposure to TCDD did not affect the percentage of intact acrosomes or even after induction of AR with progesterone (Table 3), indicating absence of TCDD-induced disruption.

3.2.3. Sperm $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ of capacitated sperm was monitored immediately after the initial contact with the medium (Figure 3). After adjusting the effect of DMSO exposure throughout time, we found that, in overall, only 4.9% and 7.5% of the cells analyzed at 1 nmol/L and 1 μmol/L, respectively, increased intracellular Ca^{2+} levels significantly above the control (Figure 3, fluorescence-time trace b, P<0.05).

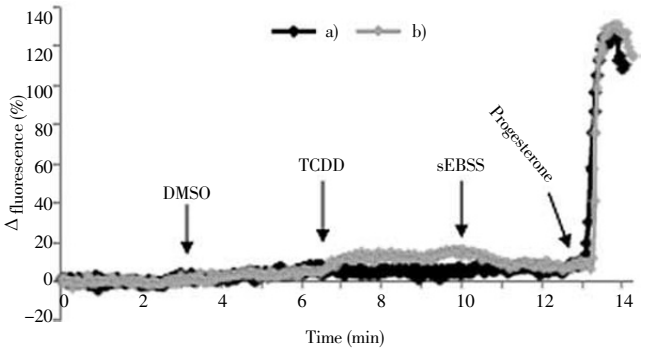

Figure 3. Fluorescence-time traces examples representing intracellular Ca^{2+} changes in a) a capacitated sperm population from a single experiment (mean value, n=100 sperm cells) and b) a single capacitated spermatozoon from the sperm population that increased its $[Ca^{2+}]_i$ above DMSO (P<0.05).
Cells were loaded with the fluorescent Ca^{2+} marker Oregon Green BAPTA-1 AM and frames were taken in every 2.5 s. Spermatozoa were bathed in a Ca^{2+}-containing medium (sEBSS) and DMSO, 1 μmol/L TCDD and 3.2 μmol/L progesterone were added at specific time points. After TCDD exposure, cells were washed with sEBSS before progesterone addition.

4. Discussion

TCDD, the golden standard dioxin, has been shown to interfere with mitochondria functionality in cancer[17], trophoblast[22] and lung cells[25], among others. For instance, TCDD-induced tumor progression was shown to be related with a decrease in mitochondrial transcription and induction of mitochondrial stress signaling[17]. Furthermore, apoptosis of TCDD-treated trophoblast cells was found to be mediated by Bax and cytochrome c released from dysfunctional mitochondria[22], whereas analysis of bioenergetics parameters of isolated lung mitochondria exposed to TCDD revealed a decreased phosphorylation capacity[25]. Although literature on the effects of TCDD on the male reproductive tract has also suggested a role of mitochondria in the overall toxicity of dioxins, as pointed in the introduction, these studies have not clarified the role of mitochondria in TCDD-induced toxicity: are mitochondria affected by the transcriptional alterations promoted by the shuttling of the AhR to the nucleus, or are instead direct targets of the compound? For that, the 24-hour time point was chosen to discriminate between mitochondria alterations attributable to a direct action of TCDD on these organelles and alterations arising from altered gene expression. It has been shown that

Table 3
Evaluation of several human sperm functional parameters after a 24-hour exposure to TCDD at 37 °C and 5% (v/v) CO_2.

Treatment	Percentage of live cells	Total motility	Percentage of capacitated sperm	Percentage of intact acrosomes	
				No progesterone	3.2 μmol/L progesterone
Control	71.3±7.2	71.0±6.2	33.8±6.6	45.5±9.2	33.8±8.9
TCDD (1 nmol/L)	70.2±7.4	69.0±6.6	34.0±7.8	46.0±9.9	36.0±9.9
TCDD (1 μmol/L)	70.2±6.1	67.8±6.2	30.0±5.2	40.8±6.5	31.0±11.3

Results represent the mean±SEM. Two hundred sperm cells were observed for each sample (n=6). P>0.05 between control and treatment groups.

mitochondrial-related genes present an altered expression after 4-5 d of TCDD exposure in Zebrafish embryos[53] and that post-translational regulation of mitochondrial proteins, such as VADC2, by TCDD may be observed after 24 h of exposure[54].

From our results, one may conclude that TCDD had no or very little direct effect on testicular mitochondria. However, a more in depth analysis of testicular mitochondria bioenergetics parameters revealed a slight alteration in the mitochondrial potential during ADP phosphorilation. In the cell context, mitochondria are always in the presence of ADP/ATP suggesting that what seemed a slight increase in the mitochondrial potential may at a low rhythm, contribute to the increased ROS production and oxidative stress along with the reduced antioxidant defenses observed by others[18,26,31,34,37]. Unfortunately, we could not measure ROS production in isolated testicular mitochondria due to low signal-to-noise ratio (data not shown). Through the use of $AhR^{-/-}$ mice, oxidative stress was shown to be mediated through the AhR[20], but the exact mechanism/pathway by which occurs, or if it is a primary cause or consequence, is not yet established.

In a recent paper, Tappenden and colleagues described the presence of AhR in the mitochondrial fraction of different cancer cell lines, and more strikingly, it was pulled down when anti-ATP5α1 was used in co-immunoprecipitation assays[55]. The same authors measured the inner MMP using the fluorescent probe TMRM and observed an increase in the MMP with increasing concentrations of TCDD, without however, observing any difference in the cell ATP concentration[55]. These data may explain our results since we observed an increase in MMP with no alteration of mitochondrial respiration or in the activity of the ATP synthase, indicating preservation of the mitochondria phosphorylative capacity. The increase in the inner MMP had been already described by Shertzer and colleagues, using isolated liver mitochondria and JC-1 fluorescence ratios[21]. However, in this cell model, the increase in MMP was accompanied with a decrease in ATP synthesis and an increase in the state 3 respiration. As previous reports from our group have demonstrated, the differences observed between the several cell/animal models in ATP production and state 3 respiration may be related to differences in mitochondrial constitution (i.e., on the types of mitochondria present) and not due to experimental design[40].

Although human sperm also expresses the AhR[41] and are capable of non-genomic signal transduction pathways[42-44], we failed to detect any difference in the percentage of sperm with high MMP or any other alteration in viability, motility, capacitation and acrosomal integrity after 24 h of TCDD treatment, even at 1 μmol/L, a concentration far higher than the ones found in reproductive tissues[56,57]. These results contradict some published reports on the effects of dioxin or dioxin-related compounds after long-term exposure to TCDD in both men[2] and animals[58,59]. However, they are in accordance with other studies using *in vitro* TCDD or related compounds exposure[60-62]. In the only paper that addressed the effect of TCDD in sperm mitochondria, Fisher *et al*. described an increased proportion of C57BL/6 mouse epididymal sperm with low MMP when the animal was injected with TCDD, and that this alteration was dependent of the AhR presence[36]. The same effect was observed in sperm treated for 45 min with TCDD, suggesting that sperm cells are directly affected by this compound[36]. Either human sperm are overall more resistant to TCDD, as suggested by the characteristics of the human AhR, more closely related to the allele present in TCDD resistant mice[39], or mitochondria from human sperm differ from the mouse sperm counterpart. Also, the methodology applied to observe human sperm MMP in this study is not able to discriminate slight increases in this parameter, rendering a direct comparison with the results obtained in rat testicular mitochondria impossible.

The process of capacitation comprises several physiological and functional alterations (e.g., protein phosphorylation) without which sperm do not undergo AR. However, little is known about the effects of environmental contaminants in such process as well as in acrosome integrity. In fact, although capacitation was increased after *in vitro* treatment with an organochlorine mixture containing mainly PCBs and DDE in boar sperm[63], our organochlorine compound did not affect human sperm capacitation detected by tyrosine phosphorylation. Furthermore, the proportion of intact acrosomes was similar in both control and TCDD-treated groups as well as after progesterone addition. In accordance, others failed to detect any difference in human sperm after PCBs, lindane, bisphenol A (BPA) and octylphenol *in vitro* exposures[61,62,64]. However, in boar[63] and human sperm[43] AR was inhibited, even after progesterone treatment[43]. In the only study using animals treated with TCDD, the authors reported a decrease in the AR rate in the presence of heparin[58].

For all the cellular events mentioned above, Ca^{2+} is thought to be an important player. Regardless their apparent straightforward structure, spermatozoa possess a refined machinery responsible for $[Ca^{2+}]_i$ regulation and production of Ca^{2+} signals, assuring that specific responses only occur at appropriate time points, therefore making possible their quest to achieve fertilization[65]. Some environmental contaminants such as lindane and a mixture of several organochlorines have been shown to interfere with this regulation, increasing $[Ca^{2+}]_i$, in both human[66] and boar sperm[63]. In our study, TCDD also induced a significant elevation of $[Ca^{2+}]_i$ but only in a very small fraction of cells from the total population, suggesting the low biological relevance of this finding. It seems therefore unlikely the involvement of Ca^{2+} signaling pathway(s) in sperm directly exposed to TCDD. Luconi and co-workers concluded the same after observing that both xenoestrogens BPA and octylphenol failed to promote any change in human sperm Ca^{2+} levels[64]. This result is, nevertheless, in agreement with the lack of effects observed in this study, particularly in AR, a strongly Ca^{2+}-dependent key event.

In conclusion, besides the effects elicited by the shuttle of the AhR to the nucleus, and although only small mitochondrial changes were observed, we suggest that TCDD may have a direct action on the mitochondria that can contribute to the alterations in spermatogenesis already described. Also, we consider that the alterations observed in men semen parameters exposed to TCDD may arise from the process of spermatogenesis or during the transit through the epididymis, since no alterations in sperm functional parameters were detected when sperm were treated directly with TCDD. It is also likely that the alterations described in semen parameters in men exposed to TCDD may result from a cumulative effect of the mixture of environmental contaminants that humans are daily exposed to.

Conflict of interest statement

We declare that we have no conflict of interest.

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